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The molecular motion of bacteriorhodopsin mutant D96N in the purple membrane

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Abstract We measured the flash-induced absorption anisotropies of mutant bacteriorhodopsin (bR), D96N, in the purple membrane suspension. The measured anisotropy decay at 410 nm differed from that at 570 nm. These wavelength-dependent anisotropies show that the motion of absorption dipole of non-excited bR is faster than that of M-intermediate. The motion of non-excited bR is considered as the rotational motion of whole protein in the purple membrane. This fact suggests that the photo-excitation induces the conformational change of the protein and/or the inter-protein interaction within the membrane, which prevents the motion of M-intermediate.

Key words: Bacteriorhodopsin; Absorption anisotropy; Molecular motion of protein; Site-specific mutant; Azide

1. Introduction

Bacteriorhodopsin (bR) is a single protein, containing a retinal Schiff base, in the purple membrane of *Halobacterium salinarium* [1]. The retinal absorbs the light and triggers the photochemical cycle [2], which accomplishes a proton translocation from inside to outside the cell. Within the purple membrane, bR molecules form trimmers, which arrange hexagonal lattice [3].

The conformational changes of the protein play important role for the proton-pumping mechanism. It seems likely that the conformational changes also relate to the wobbling motion of the retinal inside the protein and/or the rotational motion of the whole protein within the membrane. For detection of the motion, absorption anisotropy becomes a powerful tool. Using this method, however, an absence of the motion of retinal was reported [4–8]. The immobilization of the retinal was explained by the restriction with peptide chains around retinal and the rigid structure of the purple membrane. Only a few investigators observed the wavelength-dependent anisotropy changes, and suggested that the rotational motion of whole protein occurs during photochemical cycle [9–11]. In these studies, the factors, which confused the data analysis, were as follows: (1) the overlapping of absorption from ground-state bR and photo-intermediates; and, (2) in point of the motion, ground-state bR must be distinguished into non-excited bR and 'returned' bR. Here, 'returned' bR means the ground-state bR, which had been activated on flash irradiation and have finished the photochemical cycle.

To simplify these factors, in the present work, we performed the absorption anisotropy measurements for mutant bR, D96N, in which the Asp-96 of the wild-type bR is replaced with Asn. In the proton-pumping mechanism, the Asp-96 acts as the proton donor for the deprotonated Schiff base. This reprotonation step of the Schiff base is correlated to the decay of M-intermediate, and is disturbed dramatically by the replacement Asp-96 with Asn [12–15]. D96N shows a similar photochemical reaction until the formation of M-intermediate. The decay of M-intermediate was very slow by a factor of hundreds more than that of wild-type bR. During an early stage after the flash excitation, therefore, we can regard the present species as only M-intermediate and non-excited bR. This fact provides the simple condition to analyse the measured absorption anisotropies. For D96N, addition of azide accelerates the decay of M-intermediate [16]. In this situation, other photo-intermediates following M-intermediate accumulate [17]. Therefore, we also examined the effect of the appearances of the other photo-intermediates on the anisotropy change.

In this study, we will report the specific anisotropy changes of excited and non-excited bR species, and discuss the molecular motion of bR within the membrane.

2. Materials and methods

2.1. Sample preparation and chemicals

Purple membranes of D96N were isolated from mutated *H. salinarium* L-33 by the standard method [18]. All samples were the suspension of the purple membrane in a buffer solution, and the concentrations were adjusted so as to maintain the absorbance value of 0.8 at 570 nm. The buffer solution used was sodium phosphate, 10 mM, pH = 6.9.

2.2. Absorption anisotropy measurements

The source of the actinic flash (7 ns) was a second-harmonic of the fundamental beam of the Q-switched Nd-YAG laser (Quanta-Ray, DCR-2). The excitation wavelength used was 532 nm, and polarized by Glan Thompson polarizer (Optics For Research, PLU-10) placed just in front of the sample. Two monochromators were placed in rear of the monitoring light source and in front of the photomultiplier (Hamamatsu, R2949). Sheet polarizers, placed both in front and rear of the sample, were rotated 90° to change the polarized direction of monitoring light. The absorption changes at λnm, $\Delta A_\lambda^{\parallel}(t)$ and $\Delta A_\lambda^{\perp}(t)$, were obtained with the monitoring lights polarized parallel and perpendicular to the polarization of the actinic flash, alternately. Using $\Delta A_\lambda^{\parallel}(t)$ and $\Delta A_\lambda^{\perp}(t)$, the absorption anisotropies (r_λ) were calculated according to following equation:

$$r_\lambda(t) = \frac{\Delta A_\lambda^{\parallel}(t) - \Delta A_\lambda^{\perp}(t)}{\Delta A_\lambda^{\parallel}(t) + 2\Delta A_\lambda^{\perp}(t)} \quad (1)$$

All measurements were performed at 20°C.

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3. Results and discussion

In Fig. 1, solid and broken lines show measured anisotropies of D96N in purple membrane suspension at 410 and 570 nm, respectively. The anisotropy change at 570 nm was faster than that at 410 nm. In this measuring time range, the present species are M-intermediate and non-excited bR, because the decay of M-intermediate is negligible (data not shown). The wavelength-dependent anisotropy changes indicate that the absorption dipoles of M-intermediate undergo the different motion from these of non-excited bR. As M-intermediate has little absorption at 570 nm, the anisotropy change at 570 nm depends exactly on the motion of non-excited bR. At 410 nm, however, both M-intermediate and ground-state bR have absorption and contribute to the anisotropy change [19,20].

Taking account of this absorption overlapping, we calculated the anisotropy attributed exactly to M-intermediate, $r^{M}(t)$, with next equation.

$$r^{M}(t) = \frac{\Delta A_{410}(t) r_{410}(t) - \frac{\varepsilon_{410}^{bR}}{\varepsilon_{570}^{bR}} \Delta A_{570}(t) r_{570}(t)}{\Delta A_{410}(t) - \frac{\varepsilon_{410}^{bR}}{\varepsilon_{570}^{bR}} \Delta A_{570}(t)} \quad (2)$$

Here, ΔA_{λ} and r_{λ} are the induced absorption change and anisotropy at λnm, and $\varepsilon_{\lambda}^{bR}$ is the extinction coefficient of ground-state bR at λnm. The value of $\varepsilon_{410}^{bR}/\varepsilon_{570}^{bR}$ was determined as 0.22 according to the previously reported absorption spectrum [19,20]. The calculated values of $r^{M}(t)$ are plotted in Fig. 1. The slow decay of $r^{M}(t)$ is almost the same with that of measured anisotropies for wild-type bR [5,8]. The reported results were attributed to the rotational motion of purple membrane sheet itself. On the other hand, the anisotropy of non-excited bR decreased faster than that of M-intermediate. This means that, within the membrane, the absorption dipoles of non-excited bR undergo the motion. This motion is considered as the wobbling motion of the chromophore inside the protein and/or the rotational motion of whole protein within the membrane. We express the reorientational angle of the absorption dipole occurred by the rotation of membrane itself as $\Delta\theta_{m}$, and that

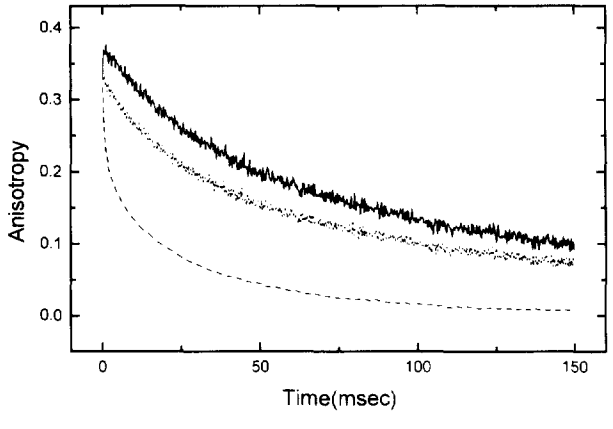

Fig. 1. Absorption anisotropies of D96N in purple membrane suspension. Solid and broken lines are measured anisotropies at 410 and 570 nm, respectively. The anisotropy at 570 nm is considered as the anisotropy of non-excited bR (r^{bR}, see text). Dotted line is the calculated anisotropy, which is attributed exactly to M-intermediate (r^{M}, see text). It is noted that the decay of r^{bR} is faster than that of r^{M}.

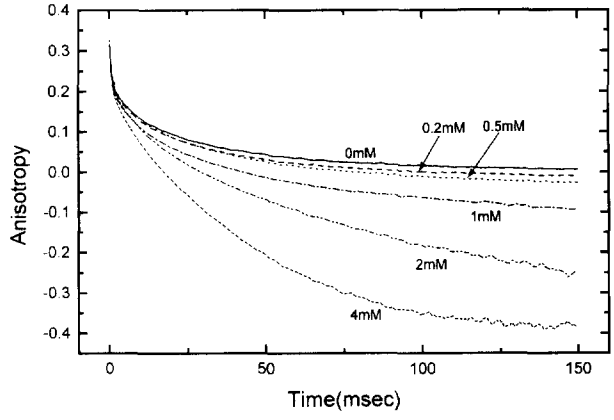

Fig. 2. Measured absorption anisotropies of D96N in purple membrane suspension in the presence of various concentrations of azide at 570 nm. The concentrations of azide are denoted in this figure. With increasing the concentration of azide, the decay of the anisotropy becomes rapid, and the anisotropy takes negative value.

occurred by the motion of the absorption dipole within the membrane as $\Delta\theta$. Then, the anisotropy of non-excited bR, $r^{bR}(t)$, can be written as follows:

$$r^{bR}(t) = r^{bR}(0) \cdot P_2(\Delta\theta_{m}) \cdot P_2(\Delta\theta) \quad (3)$$

where $P_2(\alpha) = (3\cos^2\alpha - 1)/2$ is the second Legendre polynomial, and $\Delta\theta_{m}$ and $\Delta\theta$ are the averages of all non-excited bR at time t. Because the change of $r^{M}(t)$ is considered as the rotational motion of the membrane itself, $P_2(\Delta\theta_{m})$ is expressed with $r^{M}(t)/r^{M}(0)$. In Fig. 1, the values of r^{bR} and r^{M} at $t = 0$ are 0.325, and these at 150 ms are 0.007 and 0.075, respectively. Then, $\Delta\theta$ can be calculated with Eq. 3, and the value is 51°. If this large reorientational angle were caused by the wobbling motion of the chromophore inside the protein, the absorption spectrum of ground-state bR would be different from that of wild-type bR. The wobbling angle should be constrained by the neighboring peptide chains around retinal, and the motion of peptide chains usually occurs from ps to ns [21]. Therefore, the wobbling motion of the chromophore probably makes little contribution to the anisotropy changes. The rotational motion of whole protein becomes the most feasible origin for the faster decay of r^{bR} than that of r^{M}. When the whole protein rotates around the normal to the plane of the membrane, $P_2(\Delta\theta)$ decreases as time proceeding and takes the constant value finally, expressed by $(3\cos^2\beta - 1)^2/4$ [22]. Here, β is the angle between the absorption dipole and the normal to the plane of the membrane. In bR, β is ~70° [23,24]. Using $(3\cos^2\beta - 1)^2/4$ as $P_2(\Delta\theta)$, calculated value of r^{bR} at 150 ms became near to 0 (0.008).

The nearly 0 value of the anisotropy of non-excited bR indicates that the angular distribution of the absorption dipoles in non-excited bR is almost random. In the measuring time range, on the other hand, the anisotropy of M-intermediate takes positive value, and so the absorption dipoles in M-intermediates maintain the vertically polarized angular distribution (the actinic flash is vertically polarized). For D96N, the decay of M-intermediate becomes rapid by addition of azide [16]. In this condition, other photo-intermediates (like as N and O) following M-intermediate accumulate [17]. The accumulated species have absorption at 570 nm. When the angular distributions of

these species are also vertically polarized, induced absorption change, $\Delta A^{\parallel}_{570}(t)$, increases and the value of the numerator $(\Delta A^{\parallel}_{570}(t) - \Delta A^{\perp}_{570}(t))$ in Eq. 1 becomes positive. In this situation, therefore, the anisotropy at 570 nm should become negative. Fig. 2 shows the measured anisotropies at 570 nm in the presence of various concentrations of azide. With increasing concentration of azide, the decay of anisotropy became rapid, and finally the anisotropy took negative value. This fact means that the other photo-intermediates following M-intermediate maintain the vertically polarized angular distribution at M-intermediate.

As shown in Fig. 1, the motion of M-intermediate is slower than that of non-excited bR. This fact indicates that the motion of M-intermediate is restricted with a certain mechanism. The mechanism must be triggered by the conformational change at M-intermediate. Thus, the changed conformation of M-intermediate should slow the rotational motion of the monomer protein or lead the binding between M-intermediate and neighboring proteins. For wild-type bR, whether the protein undergoes the motion in the purple membrane is still an open question [4–11]. For D96N, however, we confirmed that non-excited molecules rotate within the membrane. The replacement Asp-96 with Asn must affect the protein conformation. Therefore, this site-specific mutation probably disrupts the tight packing of the ground-state bR in the purple membrane and/or weakens the binding affinity of ground-state bR with neighboring proteins. Consequently, the motion of non-excited bR is considered to become measurable.

The change of the rotational motion of the protein is not caused by the internal conformation change around retinal. Thus, in M-intermediate, the conformation of the outer part of the protein must alter. Similar conformational change also occurs in the photoreceptor proteins. Throughout the animal world, retinylidene proteins, rodopsins, form a widespread family as a visual pigment. In *H. salinarium*, sensoryrhodopsin and phoborhodopsin are phototaxis receptors. These photoreceptors change their conformations by light absorption, and the changed conformations of the outer parts of the proteins activate the signal transducer proteins. Therefore, our observed conformational change of M-intermediate and/or induced interaction with neighboring proteins may be the intrinsic property for retinylidene proteins.

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